

Practitioner's Docket No. 49651 (1526)

PATENT

TRANSMITTAL LETTER TO THE U.S. DESIGNATED OFFICE (DO/US)--
ENTRY INTO THE U.S. NATIONAL STAGE UNDER CHAPTER I

<u>PCT/JP99/04399</u>	<u>13 August 1999</u>	<u>14 August 1998</u>
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

NUCLEIC ACID CAPABLE OF BINDING SPECIFICALLY TO Ras TARGET PROTEIN
TITLE OF INVENTION

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APPLICANT(S)

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: DO/US

NOTE: The completion of those filing requirements that can be made at a time later than 20 months from the priority date results from the Commissioner exercising his judgment under the authority granted under 35 U.S.C. § 371(d). The filing receipt will show the actual date of receipt of the last item completing the entry into the national phase. See 37 C.F.R. § 1.491, which states: "An international application enters the national stage when the applicant has filed the documents and fees required by 35 U.S.C. § 371(c) within the periods set forth in § 1.494 and § 1.495."

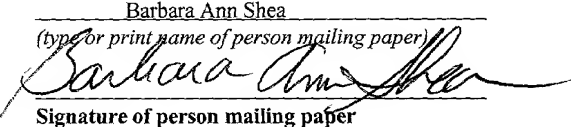
WARNING: Where the items are those that can be submitted to complete the entry of the international application into the national phase subsequent to 20 months from the priority date, the application is still considered to be in the international stage. And if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. § 1.10 must be used (because international application papers are not covered by an ordinary certificate of mailing, 37 C.F.R. § 1.8(2)(xi)).

WARNING: Documents and fees must be clearly identified as a submission to enter the national stage under 35 U.S.C. § 371, otherwise the submission will be considered as being made under 35 U.S.C. § 111, 37 C.F.R. § 1.494(f).

CERTIFICATION UNDER 37 C.F.R. § 1.10*
(Express Mail label number is **mandatory**.)
(Express Mail certification is optional.)

I hereby certify that this paper, along with any document referred to, is being deposited with the United States Postal Service on this date April 11, 2000, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number **TB553892372US**, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Barbara Ann Shea
(type or print name of person mailing paper)


Signature of person mailing paper

WARNING: Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

***WARNING:** Each paper or fee filed by "Express Mail" must have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).

"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

(Transmittal Letter to the United States Designated Office (DO/US - Entry into National Stage
under 35 USC 371--page 1 of 7)

WARNING: Failure to pay the national fee within 20 months from the priority date will result in the abandonment of the application. The time for payment of the basic fee is not extendable. M.P.E.P. § 1893.01(a)(1), 6th ed., rev. 3.

1. Applicant herewith submits to the United States Designated Office (DO/US) the following items under 35 U.S.C. 371:

- a. ☒ This express request to immediately begin national examination procedures (35 U.S.C. § 371(f)).
- b. ☒ The U.S. National Fee (35 U.S.C. § 371(c)(1)) and
☒ other fees (37 C.F.R. § 1.492), as indicated below:

2. Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
*	TOTAL CLAIMS	21 - 20 =	1	x\$ 18.00=	\$ 18.00
	INDEPENDENT CLAIMS	2 - 3 =	0	x\$ 78.00=	\$0
	MULTIPLE DEPENDENT CLAIMS(S) (if applicable) + \$260.00				\$ 260.00
	The international search fee, as set forth in § 1.445(a)(2) to be paid to the US PTO acting as an international Searching Authority:				\$ 840.00
BASIC FEE**	<input type="checkbox"/> has been paid (37 CFR 1.492(a)(2)).....\$760.00 <input type="checkbox"/> has not been paid (37 CFR 1.492(a)(3)).....\$970.00 <input checked="" type="checkbox"/> where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5)) \$840.00				
	Total of above Calculation				= \$1,118.00
	Reduction by ½ for filing by small entity, if applicable. Affidavit must be filed also. (note 37 CFR 1.9, 1.27, 1.28)				-
	Subtotal				\$1,118.00
	Total National Fee				\$1,118.00
SMALL ENTITY	Fee for recording the enclosed assignment document \$40.00 (37 CFR 1.21(h)). (See Item 10 below). See attached "ASSIGNMENT COVER SHEET (37 CFR 3.34)".				\$
	TOTAL				Total Fees enclose \$1,118.00

****WARNING:** "To avoid abandonment of the application, the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 20 months from the priority date; *** (2) the basic national fee (see § 1.492(a)). The 20-month time limit may not be extended." 37 C.F.R. § 1.494(b).

- i. ☒ A check in the amount of \$ 1,118.00 to cover the above fees is enclosed.
 ii. ☐ Please charge Account No. _____ in the amount of \$ _____.
 A duplicate copy of this sheet is enclosed.

WARNING: *If the translations of the international application and/or oath or declaration have not been submitted by the applicant within twenty (20) months from the priority date, the applicant will be so notified and given a period of time within which to file the translation and/or oath or declaration in order to prevent abandonment. The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than twenty (20) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than twenty (20) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 will apply. 37 C.F.R. § 1.494(c).*

3. A copy of the International application as filed (35 U.S.C. § 371(c)(2)):
- a. ☒ is transmitted herewith.
 - b. ☐ is not required, as the application was filed with the United States Receiving Office.
 - c. ☐ has been transmitted
 - i. ☐ by the International Bureau. Date of mailing of the application Prom form PCT/IB/308): _____.
 - ii. ☐ by applicant on _____.
- Date

NOTE: *Section 1.494(b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 20 months from the priority date to avoid abandonment. "The International Bureau nominally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies the applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage and applicant has received notice from the International Bureau, applicant need only pay the basic national fee by 20 months from the priority date." [This can now be paid subsequently with a surcharge.] Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35.*

4. A translation of the International application into the English language (35 U.S.C. § 371(c)(2)):
- a. ☒ is transmitted herewith.
 - b. ☐ is not required as the application was filed in English.
 - c. ☐ was previously transmitted by applicant on _____.
- Date

5. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3)):

NOTE: *The Notice of January 7, 1993 indicates that 37 C.F.R. § 1.494(d) was "amended to clarify the existing practice that PCT Article 19 Amendments must be submitted by 20 months from the priority date, which time may not be extended." This Notice further advises: "Of course, the failure to do so does not result in loss of the subject matter of PCT Article 19 amendments. The applicant may submit that subject matter in a preliminary amendment filed under Section 1.121. In many cases, filing an amendment under Section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 35. See item 11(c) below. See also 37 C.F.R. § 1.494(d).*

- a. ☒ are transmitted herewith.
- b. ☐ have been transmitted

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- i. ☐ by the International Bureau. Date of mailing of the amendment (from form PCT/IB/308): _____.
- ii. ☐ by applicant on _____.
- Date
- c. ☐ have not been transmitted, as
- i. ☐ no notification has been received that the International Search Authority has received the Search Copy.
- ii. ☐ the Search Copy was received by the International Searching Authority, but the Search Report has not yet been issued. Date of receipt of Search Copy from form PCT/ISA/202): _____.
- iii. ☐ applicant chose not to make amendments under PCT Article 19. Date of mailing of Search Report (from form PCT/ISA/210): 1 January 1999
- iv. ☐ the time limit for the submission of amendments has not yet expired. The amendments, or a statement that amendments have not been made, will be transmitted before the expiration of the time limit under PCT Rule 46.1.
6. ☒ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)):
- a. ☒ is transmitted herewith.
- b. ☐ is not required as the amendments were made in the English language.
- c. ☐ has not been transmitted for reasons indicated at point 5(c) above.
7. ☒ An oath or declaration of the inventor including power of attorney (35 U.S.C. § 371(c)(4)) complying with 35 U.S.C. § 115
- a. ☐ was previously submitted by applicant on _____.
- Date
- b. ☐ is submitted herewith, and such oath or declaration
- i. ☐ is attached to the application.
- ii. ☐ identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or (c) and 5(b); and states that they were reviewed by the inventor, as required by 37 C.F.R. § 1.70.
- iii. ☒ will follow.
- II. Other document(s) or information included:
8. ☒ An international Search Report or Declaration under PCT Article 17(2)(a):
- a. ☒ is transmitted herewith.
- b. ☐ has been transmitted by the International Bureau. Date of mailing from form PCT/IB/308): _____.
- c. ☐ is not required, as the application was searched by the United States International Searching Authority.
- d. ☐ will be transmitted promptly upon request.

- e. ☐ has been submitted by applicant on _____.
Date
- f. ☐ is not transmitted, as the international search has not yet issued.
9. ☒ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98:
a. ☐ is transmitted herewith.
Also transmitted herewith is (are)
☐ Form PTO-1449 (PTO/SB/08A and 08B)
☐ Copies of citations listed
b. ☒ will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. § 371(c).
c. ☐ was previously submitted by applicant on _____.
Date
10. ☐ An assignment document is transmitted herewith for recording. A separate
☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW
PATENT APPLICATION" or
☐ FORM PTO—1595 is also attached.
☐ Please mail the recorded assignment document to:
i. ☐ the person whose signature and address appears below.
ii. ☐ the following:
11. ☒ Additional documents
a. ☒ Copy of request (PCT/RO/101)
b. ☒ International Publication No. WO 00/09684
i. ☒ Specification, claims and drawing
ii. ☐ Front page only
c. ☐ Preliminary amendment (37 C.F.R. § 1.121)
d. ☐ Other:
12. ☒ The above checked items are being transmitted
a. ☐ before the 18th month publication.
b. ☒ after publication and the article 20 communication, but before 20 months from the priority date.
c. ☐ after 20 months (revival).

NOTE: Petition to revive (37 C.F.R. § 1.137(a) or (b)) is necessary if 35 U.S.C. § 371 requirements are submitted after 20 months.

13. ☐ Certain requirements under 35 U.S.C. § 371 were previously submitted by the applicant on _____ namely:
Date

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: *"A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).*

NOTE: *"Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).*

☒ The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. 04-1105.

☒ 37 C.F.R. § 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 20 months without extension (37 C.F.R. § 1.494(b)(2)), results in abandonment of the application, it would be best to always check the above box.*

☒ 37 C.F.R. § 1.492(b), (c), and (d) (presentation of extra claims)

NOTE: *Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment, prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.*

☒ 37 C.F.R. § 1.17 (application processing fees)

☐ 37 C.F.R. § 1.17(a)(1)-(5)(extension fees pursuant to § 1.136(a).

☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b)).

NOTE: *Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).*

NOTE: *37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying or at the time of paying . . . issue fee...." From the wording of 37 C.F.R. § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.*

☐ 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 20 months after the priority date.

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SIGNATURE OF PRACTITIONER

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(type or print name of practitioner)

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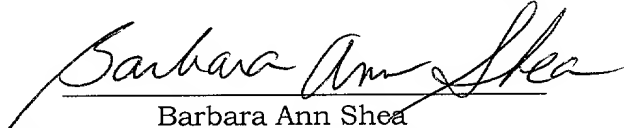
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Shigeuyki Yokoyama et al. Examiner: Not Assigned
Serial No.: Not Assigned Group: Not Assigned
Filed: Herewith
For: NUCLEIC ACID CAPABLE OF BINDING SPECIFICALLY TO Ras
TARGET PROTEIN

Certificate of Mailing

I hereby certify that this correspondence is, on the date shown below, being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to Box Amendment, Assistant Commissioner of Patents and Trademarks, Washington, D. C. 20231.

Dated: April 11, 2000


Barbara Ann Shea

BOX AMENDMENT
Assistant Commissioner of Patents and Trademarks
Washington, D. C. 20231

Sir:

PRELIMINARY AMENDMENT

Applicants file herewith the above-identified application. Please amend the application as follows:

IN THE CLAIMS:

Please cancel claims 1-18 and add the following new claims.

19. A nucleic acid capable of being specifically bound to a target protein of Ras.

20. The nucleic acid as claimed in claim 19, which nucleic acid is an RNA.

21. The nucleic acid as claimed in claim 19 or 20, which nucleic acid is specifically bound to the Ras binding domain of the target protein of Ras.

22. The nucleic acid as claimed in any of claims 19 to 21, wherein the target protein of Ras is Raf-1.

23. The nucleic acid as claimed in claim 22, which nucleic acid is an RNA that is specifically bound to a Ras binding domain (R&D) of Raf-1.

24. The nucleic acid as claimed in any of claims 20 to 22, wherein the RNA is an RNA containing at least any one of base sequences of sequence Nos. 1 to 28 of Sequence Listing or a base sequence in which at least one base thereof is deleted and substituted with another base and/or at least one base is added.

25. The nucleic acid as claimed in claim 24, wherein the RNA is an RNA containing at least any one of base sequences of sequence Nos. 1 to 8 or sequence Nos. 25 to 28 of Sequence Listing or a base sequence in which at least one base thereof is deleted and substituted with another base and/or at least one base is added.

26. A nucleic acid having a complementary base sequence to the nucleic acid as claimed in claim 24 or 25.

27. An agent for controlling cell signal transduction which agent is made of the nucleic acid as claimed in any of claims 19 to 26.

28. The controlling agent as claimed in claim 27, wherein the nucleic acid is an RNA.

29. A method of controlling cell signal transduction using the nucleic acid as claimed in any of claims 19 to 26.

30. The method as claimed in claim 29, wherein the nucleic acid is an RNA.

31. A pharmaceutical composition containing the nucleic acid as claimed in any of claims 19-26.

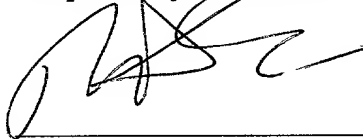
32. The pharmaceutical composition as claimed in claim 31, which composition is used for treating cancers or inflammatory diseases.

33. A method of selecting an RNA having an ability of specific binding to a target protein of Ras, which comprises selecting the RNA having the ability of specific binding to the target protein of Ras from an RNA pool having various base sequences.

34. The method as claimed in claim 33, wherein the RNA of the RNA pool having various base sequences is an RNA comprising 20 to 300 bases.

35. The method as claimed in claim 33 or 34 wherein the target protein of Ras is Raf-1.

Respectfully submitted,



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Dated: 4/11/00

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NUCLEIC ACID CAPABLE OF BINDING SPECIFICALLY TO Ras TARGET
PROTEIN

Technical Field

The present invention relates to novel nucleic acids (aptamer) which are specifically bound to target proteins of Ras. More preferably, the present invention relates to novel RNA aptamers which are specifically bound to Raf-1. Further, the present invention relates to control of signal transduction that induces proliferation or differentiation of cells using the nucleic acids of the present invention, and to a pharmaceutical composition using the same.

Background of the Invention

Ras is a guanine nucleotide binding protein, and participates in signal transduction of cells. When a receptor of cells is activated, "GDP binding Ras" in cells becomes "GTP binding Ras".

This "GTP binding Ras" is bound to "target proteins of Ras" such as Raf-1, B-Raf, RGL, Ral GDS, MEKK, Pl3K and the like. These "target proteins of Ras" have a Ras binding domain (RBD) to which the GTP binding Ras can be bound, and the GTP binding Ras is bound to this domain of these "target proteins of Ras" to transmit necessary signals into cells.

Ras is a key protein of intracellular signal transduction,

and the "target proteins of Ras", such as Raf-1, are a center of the intracellular signal transduction system in which signals from Ras are transmitted according to the types.

Accordingly, a substance capable of specifically blocking the binding domain with the GTP binding Ras in the "target proteins of Ras", if any, can specifically inhibit an intracellular signal transduction system by Ras, and it is useful to treat or prevent various diseases triggered by the signal transduction. For example, with respect to tumor cells, proliferation or differentiation of tumor cells can be inhibited by specifically controlling the signal transduction that induces proliferation or differentiation with the "target cells of Ras" to treat cancers or inhibit metastasis.

By the way, Ras-1, one of the "target cells of Ras" is a serine/threonine protein kinase present in a cytoplasm, and the activity is induced by interaction with the GTP binding Ras. The activated Raf-1 phosphorylates MEK (MAPK/ERK kinase), and then MEK phosphorylates ERK to transmit signals into a nucleus (Daum, G., et al., (1994) Trends Biochem. Sci. 19, 474 - 480; Avruch, J., et al., (1994) Trends Biochem. Sci. 19, 279 - 283).

In order to elucidate such an intracellular signal transduction system of Raf-1, a method of selectively inhibiting the function of Ras or Raf-1 has been utilized (deVries-Smits, A. M., et al., (1992) Nature 357, 602 - 604). These studies include inhibition of the Ras function with a

Raf-1 mutant free from a kinase activity (Kolch, W., et al., (1991) Nature 349, 426 - 428), inhibition of a Raf-1 kinase with an antibody bound to a kinase domain of Raf-1 (Kolch, W., et al., (1996) Oncogene 13, 1305 - 1314) and the like.

However, these inhibitors do not specifically inhibit a specific part of a signal transduction system with Ras or Raf-1, but inhibit many functions such as a function of binding to Ras, a kinase function and the like simultaneously and diversely. Accordingly, a signal transduction system to be inhibited cannot be specified. Thus, individual specific mechanisms of a signal transduction system could not be clarified satisfactorily.

Consequently, the development of a molecular seed capable of specifically inhibiting the binding of Ras to Raf-1 has become important for clarifying the role of the signal transduction system.

At present, a downstream signaling pathway of Ras has not been completely clarified. When such a molecular seed is developed, it is possible to elucidate the signaling pathway in which Ras participates using a molecular seed capable of specifically inhibiting some specific routes and clarify the signaling pathway with target proteins of Ras in detail. In addition, it is possible to control the intracellular signal transduction. Consequently, various diseases in which the intracellular signal transduction participates, such as tumors

and the like, can be treated and prevented.

Meanwhile, the structural analysis of the "target proteins of Ras" in the intracellular signaling pathway in which Ras participates has been conducted. It has been known that the Ras binding domain (RBD) of Raf-1 is located from 51 to 131 residues in the N-terminus of Raf-1 (Vojtek, A. B., et al., (1993) Cell 74, 205 - 214; Chuang, E., et al., (1994) Mol. Cell. Biol. 14, 5318 - 5325).

Further, nucleic acid molecular seeds (aptamers), such as an RNA, a DNA and the like, having a high affinity for a certain target, such as proteins, have been isolated by "in vitro selection" methods (Ellington, A. D. et al., (1990) Nature 346, 818 - 822; Tuerk, C. et al., (1990) Science 249, 505 - 510) (Bock, L. C., et al., (1992) Nature 355, 564 - 566; Qiu Qiu, Y. L., et al., (1994) Nucleic Acids Res. 22, 5229 - 5234; Gal. S. W., et al., (1998) Eur. J. Biochem. 252, 553 - 562; Bell, S. D., et al., (1998) J. Biol. Chem. 273, 14309 - 14314). Therefore, there is a possibility that an RNA specifically bound to Raf-1 is obtained by applying this method to Raf-1, while the interaction with an RNA is unknown.

Disclosure of the Invention

The present invention is to provide nucleic acid molecular seeds which can specifically inhibit the binding to "GTP binding Ras" by being specifically bound to a Ras binding domain (RBD)

of target proteins of Ras such as Raf-1, B-Raf, RGL, Ral GDS, MEKK, Pl3K and the like.

In order to clarify the signaling pathway in which the target proteins of Ras participate and the physiological activity provided by the inhibition of the signal transduction, the development of substances which specifically inhibit the binding to Ras having an important role in cells in particular along with Raf-1 by being specifically bound to the target proteins of Ras, in more detail, Raf-1 and which have a strong activity have been in demand. The present inventors have found that a nucleic acid molecular seed specifically bound to the Ras binding domain of the "target proteins of Ras" can be obtained using the in vitro selection method. For example, it has been possible to obtain a novel RNA aptamer targeting the Ras binding domain (RBD) of Raf-1, one of the "target proteins of Ras" by this method and to determine the RNA sequence thereof. This RNA aptamer can specifically inhibit the binding between Ras and Raf-1.

Accordingly, the present invention is to provide novel nucleic acid molecular seeds bound to a "target protein of Ras", especially, its Ras binding domain (RBD), an agent for controlling a signal transduction system using the nucleic acid molecular seeds, a method of controlling the same, and a pharmaceutical composition containing the same.

More specifically, the present invention is to provide

RNA aptamers that bound to a "target protein of Ras", especially its RBD, an agent for controlling a signal transduction system using this RNA aptamer, a method of controlling the same, and a pharmaceutical composition containing the same.

Brief Description of the Drawings

Fig. 1 shows a sequence of an initial RNA pool in the in vitro selection of the present invention and sequences of PCR primers.

Fig. 2 shows 24 sequences of RNAs obtained from the RNA pool in the 21st round. In the drawing, a random sequence moiety of approximately 60 bases is shown. The overall sequence includes the sequences of the 5'- and 3'-termini defined in Fig. 1. Sequences which are the same as that of clone 1 are termed "group 1". The other sequences are termed "group 2". Incidentally, in note (a), the number of clones of ligands isolated respectively is shown in parentheses. In note (b), clone 21.08 indicates that each sequence defined has two mutations. In note (c), percentage of the binding of the RNA ligand to GST-RBD is based on a value measured by the nitrocellulose filter binding assay.

Fig. 3 shows the binding of the RNA ligand to the GST-RBD protein. Percentage of the binding of the RNA ligand to GST-RBD is based on a value measured by the nitrocellulose filter binding assay. In Fig. 3, a closed circle indicates the

use of an RNA of sequence No. 1, a closed square the use of an RNA of sequence No. 7, and a closed triangle the use of an RNA of sequence No. 11.

Fig. 4 is a photo that replaces a drawing, showing inhibition of interaction between Ras and GST-RBD by the RNA aptamers. An amount of the Ras protein bound to GST-RBD as measured by immunoblotting with anti-Ras antibody RAS004 is shown on an upper column. An total amount of GST-RBD measured by staining with Coomassie Blue is shown on a lower column. GDP binding (D) or GTPγS binding (T) Ras (2 pmols) and GST-RBD (25 pmols) were incubated in the presence of an RNA in various amounts. A used 21.01 ligand in Fig. 2, B 21.07 ligand in Fig. 2, C 21.11 ligand in Fig. 2, and D 21.12 ligand in Fig. 2.

Best Mode for Carrying Out the Invention

The "target proteins of Ras" of the present invention refers to Ras proteins which participate in the cell signal transduction, preferably a group of proteins forming an intracellular signal transduction system by interacting with GTP binding Ras proteins. Examples of the "target proteins of Ras" of the present invention include Raf-1, B-Raf, RGL, Ral GDS, MEKK, P13K and the like. However, this invention is not limited within the target. The "target proteins of Ras" of the present invention are preferably Raf-1 and the like.

The present invention has clarified that nucleic acid

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molecular seeds capable of being specifically bound to the foregoing "target proteins of Ras" exist. Accordingly, the nucleic acid specifically bound to the "target proteins of Ras" in the present invention may be an RNA or a DNA. The RNA or the DNA is not particularly limited so long as it is specifically bound to the "target proteins of Ras". Further, the nucleic acid of the present invention may be specifically bound to only one "target protein of Ras" or to two or more "target proteins of Ras".

The size of bases of the nucleic acid molecular seed of the present invention is not particularly limited so long as it is sufficient to allow the specific binding to the "target proteins of Ras". It is between 20 and 300 bases, preferably between 20 and 150 bases, more preferably between 30 and 150 bases, further preferably between 50 and 150 bases. In case the binding specificity is stressed, the longer size is preferable. However, in view of the ease of the procurement in the synthesis method or the like, the shorter size is preferable.

As the nucleic acid molecular seed of the present invention, an RNA containing any one of base sequences, sequence Nos. 1 to 28, preferably sequence Nos. 1 to 8 or sequence Nos. 25 to 28 of Sequence Listing is mentioned.

The RNAs of the present invention shown in the sequence numbers have an ability of binding to the "target proteins of

Ras". More specifically, the RNAs are characterized in that they are specifically bound to the Ras binding domain (RBD) of Raf-1, and the nucleic acid molecular seed of the present invention is not limited to the base sequences shown in the foregoing sequence Nos. 1 to 28. A seed having a base sequence in which at least one base of sequence Nos. 1 to 28 of Sequence Listing is deleted and substituted with another base and/or another base is added is also available so long as it has an ability of binding to the "target proteins of Ras".

The nucleic acid molecular seed of the present invention can be one containing these base sequences in the whole or a part of the molecule. For example, a base sequence or another molecular seed may further be added to the nucleic acid molecular seed having the base sequences shown in sequence Nos. 1 to 28 of Sequence Listing unless the ability of binding to the "target proteins of Ras" is inhibited.

These RNAs of the present invention can also be reversely transcribed, as required, into DNAs having complementary base sequences to the RNAs. Accordingly, the present invention relates to nucleic acid molecular seeds such as RNAs, DNAs and the like, containing any one of base sequences of sequence Nos. 1 to 28 of Sequence Listing or a base sequence in which at least one base thereof is deleted and substituted with another base and/or at least one base is added.

The "aptamer" in the present invention refers to a nucleic

acid molecular seed capable of being bound to a specific domain of a protein, and the nucleic acid may be an RNA or a DNA. An aptamer made of an RNA is called an "RNA aptamer". Accordingly, in the present invention, the nucleic acid molecular seed in the present invention is also termed an "aptamer". When the nucleic acid is an RNA, it is called an "RNA aptamer".

Incidentally, RNAs comprising approximately 60 bases shown in sequence Nos. 29 to 52 of Sequence Listing indicate base sequences comprising approximately 60 bases in a central portion of RNAs shown in sequence Nos. 1 to 24. Further, RNAs comprising approximately 45 bases shown in sequence Nos. 53 and 54 of Sequence Listing indicate base sequences comprising approximately 45 bases in a central portion of RNAs shown in sequence Nos. 25 to 28. Moreover, sequence Nos. 55 to 60 of Sequence Listing show base sequences of primers used in specific examples of the present invention.

The nucleic acid (aptamer) of the present invention can be produced by various methods. When the base sequence of the aptamer is known, it can be synthesized.

When the base sequence of the aptamer of the present invention is unknown, the aptamer can be produced through selection by the known "in vitro selection" method (Ellington, A. D. et al., (1990) Nature 346, 818 - 822; Tuerk, C. et al., (1990) Science 249, 505 - 510). The "in vitro selection" method in the present invention is described.

First, RNAs containing a random base sequence of 20 to 300 bases, preferably 30 to 100 bases, more preferably 30 to 70 bases are produced. These RNAs are produced by transcription from synthetic DNAs containing a random sequence.

A base sequence which is to be a primer in the PCR method is added to the 5'-terminus and the 3'-terminus of the DNAs. In this case, the primer is not particularly limited. A primer having a sequence of cleavage with a restriction endonuclease so as to be able to cleave this primer portion later is preferable. A size of the primer portion is not particularly limited. It is approximately between 20 and 50 bases, preferably between 20 and 30 bases. Further, the primer at the 5'-terminus may be designed such that a promoter sequence of a T7 RNA polymerase is added thereto to enable the transcription reaction from DNA to RNA.

In this manner, the RNA group (RNA pool) having the base sequences as the primer at both termini and the random base sequence in the center is produced by transcription of the DNA.

Subsequently, the RNA in this RNA pool and the "target protein of Ras", for example, Raf-1 or a peptide comprising its binding domain are contacted to separate the RNA bound to the "target protein of Ras". The selected RNA is converted to a cDNA through reverse transcription, and it is amplified by PCR using the primers. The DNA amplified is transcribed into an RNA, and this is returned to the RNA pool.

One cycle, termed a "round", comprises binding with the "target protein" of Ras in the RNA pool, the separation of the bound RNA, reverse transcription, amplification by PCR and transcription of the DNA. That is, one round means that the foregoing round is conducted once.

When the foregoing round using the RNA pool is conducted, the amount of the RNA bound to the "target protein of Ras" in the RNA pool is increased, and further the amount of the RNA having the specific binding base sequence is increased, so that the RNA to be specifically bound can be selected by repeating the round.

Such a round is conducted 5 to 50 times, preferably 5 to 30 times.

The RNA sequences selected by the "in vitro selection" method as described above are determined by a usual method, and this RNA can also be converted to a cDNA through reverse transcription by a usual method. Further, the primer regions can be cleaved as required. In this manner, the aptamers of the present invention can be obtained.

The "in vitro selection" method of the present invention is described in more detail by using Raf-1 as the "target protein of Ras".

The present inventors have prepared a pool of RNAs having a random sequence of approximately 60 bases to select RNAs bound to the Ras binding domain (RBD) of Raf-1. And, the base

sequences shown in Fig. 1 were bound to the 3'-terminus and the 5'-terminus of these RNAs. It was presumed that approximately 8×10^{13} sequences of RNAs are present in this RNA pool.

Before conducting the selection of the aptamer, the effect of the salt concentration relative to the binding between the RNA pool and Raf-1-RBD was examined. At a low salt concentration, the RNA was non-specifically bound to Raf-1-RBD. However, it was found that the non-specific binding is suppressed by increasing the salt concentration (up to approximately 150 mM). Thus, the present inventors used a phosphate buffer (hereinafter referred to as a "binding buffer") containing 137 mM sodium chloride as a buffer for the selection.

The selection from the 1st to 13th rounds was conducted by the binding between a fusion protein of glutathione S-transferase (GST) and a peptide (RBD) of 51 to 131 of Raf-1 (hereinafter referred to as "GST-RBD") and RNAs using a glutathione-Sepharose 4B matrix. In the 13th round, the binding ability (binding ratio) to the RNA pool was slightly changed from 0.16% at the initial stage to 0.36%.

Successively, the selection using a nitrocellulose filter instead of the matrix was conducted eight times (8 rounds). In the 21st round, the binding ability to the RNA pool was 22%, and the K_d value of the pool relative to the protein GST-RBD was 290 nM.

The sequences of 33 clones were determined from the RNA pool which finished the 21st round. Consequently, 24 different sequences were obtained. The sequences of the portions comprising approximately 60 bases in the 24 sequences of clones are shown in Fig. 2. They were roughly divided into two types, one having a high homology among the sequences (this group is called "group 1") and the other among which no homology, was observed (these are referred to as "group 2").

The sequences of the total sizes (approximately 100 bases) of the 8 types of RNAs (21.01 to 21.08 in Fig. 2) in group 1 are shown in sequence Nos. 1 to 8.

The interaction between the 10 RNAs among them and GST-RBD was examined by a binding assay using a nitrocellulose filter. The results are shown in right column of Fig. 2 in terms of RNA binding (%). As a result, the RNAs in group 1 showed the satisfactory binding to GST-RBD, whereas the RNAs in group 2 did not show the satisfactory binding. The K_d values of the RNAs shown in sequence Nos. 1 (21.01 in Fig. 2) and 7 (21.07 in Fig. 2) were both 300 nM. Meanwhile, that of the RNA in sequence No. 11 (21.11 in Fig. 2) was a micromol order (refer to Fig. 3). Fig. 3 shows percentages of binding to GST-RBD when using RNAs having sequence Nos. 1 (closed circle), 7 (closed square) and 11 (closed triangle) at various concentrations (nM).

Further, the RNA ligands were not bound to GST itself.

This indicates that these RNA is bound to the RBD moiety of GST-RBD rather than to the GST moiety.

It was then examined whether the RNA aptamers in group 1 inhibit the interaction between Ras and RBD (refer to Fig. 4). The RNAs having sequence Nos. 1 (A in Fig. 4), 7 (B in Fig. 4), 11 (C in Fig. 4) and 12 (D in Fig. 4) (corresponding to 21.01, 21.07, 21.11 and 21.12 in Fig. 2, respectively) were tested at concentrations of 0 to 12.5 μ M. These were incubated with GST-RBD supported on a Sepharose matrix and Ras in GTP γ S or GDP. In the presence of the RNAs (lanes 3, 4 and 5 in Fig. 4; lane 3 is 20-pmol RNA, lane 4 200-pmol RNA and lane 5 2,000-pmol RNA) or in the absence of the RNAs (lanes 1 and 3 in Fig. 4; lane 1 was in the presence of GDP and lane 2 in the presence of GTP), the binding between GST-RBD and Ras was examined by immunoblotting with anti-Ras antibody RAS004.

In Fig. 4, "Ras" indicates Ras bound to GST-RBD, and "GST-RBD" indicates as a background that GST-RBD is solely present. As stated earlier, the RNA of sequence No. 12 (D in Fig. 4) which is scarcely bound to GST-RBD did not inhibit the binding of Ras to GST-RBD even at the concentration of 12.5 μ M. This was the same with the RNA of sequence No. 11 (C in Fig. 4) in which the k_d value was a micromol order.

On the other hand, the RNAs of sequence Nos. 1 (A in Fig. 4) and 7 (B in Fig. 4) in group 1 effectively inhibited the interaction between Ras and RBD. The reason is considered to

be that these RNAs were bound to RBD. And, the k_d value of the GTP binding Ras and RBD of Raf-1 is 18 nM (Hermann, C., et al., J. Biol. Chem., 270, 2901 - 2905 (1995)), and the RNAs of sequence Nos. 1 and 7 have the binding ability which is 10 times lower than that. Despite this, these RNAs inhibit the interaction between Ras and Raf-1.

Since these RNAs do not have an affinity for Ras or a Sepharose matrix, there is no possibility that these RNAs are bound to Ras or the Sepharose matrix to inhibit the binding between GST-RBD and Ras on the matrix. This fact proved the specific binding of these RNA aptamers to RBD.

Another in vitro selection was conducted by using a double-stranded DNA pool obtained by synthesizing a single-stranded DNA pool (200 pmols, 1.2×10^{14} molecules) having a sequence of 5'-ggtaa tacga ctcac tatag ggagt ggagg aattc atcga ggcac-3' at the 5'-terminus and 5'-catat gcctt agcga cagca agctt ctgc-3' at the 3'-terminus and containing random 45 bases in the middle thereof and converting this single-stranded DNA pool to the double-stranded DNA pool by PCR.

Consequently, novel RNA aptamers to be bound to Raf-1 RBD could be obtained. These sequences are as follows, and shown in sequence Nos. 25 to 28 of Sequence Listing.

Sequence 25

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gggaguggag gaaucaucg aggcuaugu cgacuccguc uuccuucaaa ccaguauaa 60
auugguuua gcuaugccu uagcgacagc aagcuucugc 100

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Sequence 26

gggaguggag gaauucaucg aggcaugacc ucccugggca guagggguaa aaauuauuu 60
ccuacacuuc ucaugccuua ggcacagcaa gcuucugc 98

Sequence 27

gggaguggag gaauucaucg aggcauauu cgacuccguc uuccuucaaa ccaguauaaa 60
auugguuuuu gcauauccu uagcgacagc 90

Sequence 28

gggaguggag gaauucaucg aggcauauu cgacuccguc uuccuucaaa ccaguauaaa 60
auugguuuuu gcauauccu 80

The Kd values of the RNAs shown in sequence Nos. 25, 26 and 28 among these RNAs and GST-RBD were as follows.

RNA of sequence 25: 124 nM

RNA of sequence 26: 295 nM

RNA of sequence 28: 176 nM

And, the RNAs of sequences 25 to 28 all inhibited the binding between Ras and Raf-1 RBD depending on the amounts.

Sequence Nos. 25, 27 and 28 are different in the size of the 3'-terminus. From this fact, it is presumed that the RNAs of 99 to 81 bases (90 bases correspond to sequence 27) up to sequence 28 through the decrease by each one base from the 3'-side of sequence 25 also have the activity.

Sequences of 45 bases corresponding to the random region were shown in sequence Nos. 53 and 54.

The RNA aptamers of sequences 25 to 28 obtained here can be provided through transcription from synthetic DNAs or

through synthesis.

These RNA aptamers have the stronger binding activity and are made of the smaller number of RNA bases than the above-described RNA aptamers comprising approximately 60 bases. Accordingly, these are considered to be more profitable RNA aptamers.

An antibody bound to Raf-1 mutant free from a kinase activity or a kinase domain of Raf-1 has been used to study the role of Ras or Raf-1 in the cell signal transduction system (Kolch, W., et al., (1991) Nature 349, 426 - 428; Kolch, W., et al., (1996) Oncogene 13, 1305 - 1314).

The Raf-1 mutant capable of being bound to Ras without having the kinase activity not only inhibits the Ras-dependent Raf-1 activity but also blocks the wide-ranging signal transduction systems including Ras. This is because the mutant inhibits the binding of Raf-1 and also has an influence on various downstream effectors of GTP-binding Ras.

Likewise, a monoclonal antibody bound to an epitope of a kinase domain of Raf-1 inhibits all signal transduction systems including Raf-1. This is because Raf-1 is activated not only with GTP binding Ras but also through a route having no bearing on Ras (Kolch, W., et al., (1996) Oncogene 13, 1305 - 1314).

From this standpoint as well, it is said that the RNA aptamer to RBD in the present invention can specifically inhibit the binding between Ras and Raf-1 without having any effect on

the kinase activity of Ras or Raf-1 by the other signaling pathways.

Further, the RNA aptamers of the present invention can be expressed within cells (Good, P. D., et al., (1997) Gene Ther. 4,45-54), and can be applied to a wide-ranging field.

Thus, the RNA aptamers of the present invention specifically block RBD of "target proteins of Ras", more preferably Raf-1. Not only it can be used in an agent for controlling intracellular signal transduction or a method of controlling cell signaling pathway, but also it is especially suited for the field of treatment, prevention or diagnosis of various diseases in which the signal transduction system participates.

When the nucleic acid molecular seed of the present invention is used in controlling the cell signal transduction system, the nucleic acid of the present invention may directly be introduced into desired cells. It can also be introduced into cells by being inserted into viruses or the like.

Further, it is also possible that the RNA is introduced not directly but in the form of a DNA. When the nucleic acid molecular seed of the present invention is used as a pharmaceutical composition, it can parenterally be administered as such, or it can be administered by being inserted into viruses or various vectors in the form of a DNA. In these administration forms, the pharmaceutical composition

can also be provided using a pharmaceutically acceptable carrier.

The pharmaceutical composition of the present invention is useful for treatment, prevention or diagnosis of various diseases in which the cell signal transduction system participates, especially malignant tumors and inflammatory diseases.

Examples

The present invention is illustrated more specifically with reference to the following Examples. However, the present invention is not limited to these Examples.

Example 1 (Purification of a protein)

Glutathione S-transferase (GST) and a fusion protein of RBD (51 to 131 amino acid moiety of Raf-1) and GST (hereinafter referred to as GST-RBD) were expressed in E. coli strains BL21 and BL21DE3 respectively, and purified by chromatography using a column of glutathione-Sepharose 4B (made by Amersham Pharmacia Biotec) and HQ proth (perceptive) (Shirouzu M., et al., J. Biol. Chem., 273, 7737 - 7742 (1998)).

As wild type Ha-Ras protein, a protein obtained from E. coli strain BL21 was purified by column chromatography using DEAE-Sephacell, Sephadex G-75 and Resource QFPLC (made by Amersham Pharmacia Biotec) (Shirouzu M., et al., J. Biol. Chem., 273, 7737 - 7742 (1998); Shirouzu M., et al., Oncogene, 7, 475

- 480 (1992); Ito, Y., et al., Biochemistry, 36, 9109 - 9119 (1997)).

The purity of these proteins was identified through SDS-PAGE by staining with Coomassie Blue and/or silver. The purified protein was stored in 50% glycerol at -30°C.

Example 2 (In vitro selection)

A pool of DNAs containing random 60 bases was prepared. These DNAs have sequences 5'-GCCGGAATTCTAATACGACTCACTATAGGGAGATCAGAATAAACGCTCAA-3' and 5'-TTCGACATGAGGCCCTGCAGGGCG-3' at both termini for in vitro transcription and amplification by PCR.

These RNAs were heated at 75°C for 3 minutes, then ice-cooled, and incubated in a binding buffer (5 mM MgCl₂-containing phosphate buffer physiological saline solution) containing GST-RBD and glutathione-Sepharose 4B beads. The RNAs having GST-RBD bound thereto were recovered with glutathione-Sepharose beads. The beads were cleaned with a cleaning buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl₂ and 150 mM NaCl), and the RNAs on the beads were eluted with boiling water.

The 1st through 13th rounds were conducted in this manner, provided that in order to remove the RNAs bound to GST and/or the beads alone, the RNAs were passed through glutathione-Sepharose beads having supported thereon GST only before the incubation of GST-RBD (3rd to 13th rounds) and after the elution

(7th to 13th rounds).

The eluate was subjected to reverse transcription, and amplified through PCR. The RNA pool for the next round was prepared through in vitro transcription from the cDNA amplified. The 13th round and those following for selection were conducted by a filter binding method. The RNA pool (heated at 75°C for 3 minutes, and cooled to room temperature) and GST-RBD were incubated in a binding buffer at 37°C for 1 hour. The RNAs bound to GST-RBD were separated by being bound to a nitrocellulose filter. The RNAs were eluted with a buffer containing 7 M urea (Hirao, I., et al., Mol. Diversity, (under printing)). In order to remove the RNAs bound to the filter, the RNAs were passed through the filter before the amplification.

Example 3 (Nitrocellulose filter binding assay)

RNAs were obtained through the in vitro transcription with a T7 RNA polymerase using [α -³²P]UTP. The RNA (0.8 μ M) and its protein were incubated in 50 μ l of a binding buffer at 37°C for 1 hour. A part (50 μ l) of the solution was moved on a filter, and cleaned three times with 200 μ l of a cleaning buffer.

In order to determine a dissociation constant, 1.6 nM of the RNA of which the 5'-terminus was labeled with [γ -³²P]ATP and GST-RBD at various concentrations were incubated. The radiation dose on the filter was measured using a Fuji BAS2500 bio-imaging analyzer.

Example 4 (Inhibitory activity)

One microgram of GST-RBD in 160 μ l of a binding buffer containing 0.05% Triton X-100 was mixed with 10 μ l of a glutathione-Sepharose bead suspension in a phosphate buffer physiological saline solution. The mixture was incubated at 4°C for 30 minutes. After the gentle centrifugation, the supernatant liquid was discarded. A binding buffer solution containing 40 ng of Ras (this is bound through GTP γ S or GDP as described in a literature (Koide, H., et al., Proc. Natl. Acad. Sci. USA, 90, 8683 - 8686 (1993) and 160 μ l of the RNA was added to the remaining beads, and incubated at 4°C for 30 minutes. After the incubation, the beads were cleaned with 500 μ l of a cleaning buffer. The bound protein was eluted from the beads through separation using a Laemmli's buffer, and subjected to 15% SDS-PAGE. The product was subjected to immunoblotting with anti-Ras antibody RAS004 (Moodie, S. A., et al., Science, 260, 1658 - 1661 (1993)), and visualized with an ECL immune detector (manufactured by Amersham Pharmacia Biotech).

Example 5 (Formation of an RNA pool)

A single-stranded DNA (200 pmols, 1.2×10^{14} molecules) containing random 45 bases and having a sequence 5'-ggtaa tacga ctcac tatag ggagt ggagg aattc atcga ggcat-3' at the 5'-terminus and a sequence 5'-catat gcctt agcga cagca agctt ctgc-3' at the

3'-terminus was subjected to PCR using 2 primers, 5'-ggtaa tacga ctcac tatag ggagt ggagg aattc atcg-3' and 5'-gcaga agctt gctgt cgcta aggc-3', and then transcribed with a T7 RNA polymerase to form a first RNA pool.

Example 6 (Selection of RNA bound to Raf-1RBD)

Three micromols (1,800 pmols) of the RNA pool heated at 75°C for 3 minutes and then ice-cooled and 1 μ M (600 pmols) of GST-RBD were incubated in 600 μ l of a binding buffer at 37°C for 1 hour. The culture was filtered with a nitrocellulose filter, and the filter was cleaned three times with 300 μ l of a cleaning buffer. Thereafter, the RNAs on the filter were eluted with a buffer containing 7 M urea. After the reverse transcription, PCR was conducted at 12 cycles.

The reagents used here are as follows.

GST-RBD: A fusion protein of RBD (51 to 131 amino acid moiety of Raf-1) and glutathione-S-transferase is described in a literature (Shirouzu, M., et al., (1998) J. Biol. Chem. 273, 7737 - 7742).

binding buffer: 5 mM $MgCl_2$ -containing phosphate buffer
physiological saline solution

cleaning buffer: 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$ and 150 mM NaCl

Example 7 (RNAs of sequence Nos. 25 and 26)

Selection of RNAs bound to Raf-1 RBD from the first RNA pool in Example 6, reverse transcription of RNAs to DNAs, amplification and transcription of DNAs to RNAs were repeated 10 times to obtain RNAs of sequence Nos. 25 and 26 of Sequence Listing.

Example 8 (RNAs of sequence Nos. 27 and 28)

DNAs which had a complementary sequence of an RNA of sequence No. 25 and of which the 3'-terminus side was shortened were obtained by PCR using a primer 5'-ggtaa tacga ctcac tatag ggaggt ggagg aattc atcg-3' and a primer 5'-gctgt cgcta aggca tatgc taaaa c-3' or 5'-aggca tatgc taaaa ccaat ttata ac-3'. From these DNAs, RNAs of sequence Nos. 27 and 28 of Sequence Listing were obtained.

Example 9 (Cloning and determination of a sequence)

A DNA was cloned using a TOPO TA cloning kit, and the sequence was determined with an automated DNA sequencer.

Example 10 (Measurement of a Kd value)

An RNA (4 nM) of which the 5'-terminus was labeled and 50 to 1,250 nM of GST-RBD were incubated in 600 μ l of a binding buffer at 37°C for 30 minutes. The culture was filtered with a nitrocellulose filter, and the radioactivity on the filter was measured. The Kd value was calculated using a software:

Kalleider Graph (Bell, S. D., et al., (1998) J. Biol. Chem. 273, 14309 - 14314).

Example 11 (Binding inhibition experiment)

GST-RBD (20 pmols) in 160 μ l of a binding buffer containing 0.05% Triton X-100 and 10 μ l of a phosphate buffer physiological saline solution containing glutathione-Sepharose 4B beads were mixed, and the mixture was incubated at 4°C for 30 minutes. The beads were separated, and incubated with 20 pmols of Ras and an RNA (0, 20, 100 and 200 pmols) in 160 μ l of a binding buffer (5 mM MgCl₂-containing phosphate buffer physiological saline solution) at 4°C for 30 minutes. After the beads were cleaned, the bound protein was eluted with a Laemmli's buffer, subjected to SDS-PAGE, then immunoblotted using anti-Ras antibody RAS004 (Kanai, T., et al., (1987) Jpn. J. Cancer Res. 78, 1314 - 1318), and visualized with an ECL immune detector.

In the RNAs of sequence Nos. 25 to 28, the decrease in the amount of Ras was observed according to the RNA amount.

Industrial Applicability

The present invention provides RNAs which are specifically bound to target proteins of Ras such as Raf-1 and the like and which further inhibit the binding to Ras, and a method of specifically inhibiting an intracellular signaling pathway using these RNAs. The present invention can not only clarify

CLAIMS

1. A nucleic acid capable of being specifically bound to a target protein of Ras.

2. The nucleic acid as claimed in claim 1, which nucleic acid is an RNA.

3. The nucleic acid as claimed in claim 1 or 2, which nucleic acid is specifically bound to a Ras binding domain of the target protein of Ras.

4. The nucleic acid as claimed in any of claims 1 to 3, wherein the target protein of Ras is Raf-1.

5. The nucleic acid as claimed in claim 4, which nucleic acid is an RNA that is specifically bound to a Ras binding domain (RBD) of Raf-1.

6. The nucleic acid as claimed in any of claims 2 to 4, wherein the RNA is an RNA containing at least any one of base sequences of sequence Nos. 1 to 28 of Sequence Listing or a base sequence in which at least one base thereof is deleted and substituted with another base and/or at least one base is added.

7. The nucleic acid as claimed in claim 6, wherein the RNA is an RNA containing at least any one of base sequences of sequence Nos. 1 to 8 or sequence Nos. 25 to 28 of Sequence Listing or a base sequence in which at least one base thereof is deleted and substituted with another base and/or at least one base is added.

8. A nucleic acid having a complementary base sequence to the nucleic acid as claimed in claim 6 or 7.

9. An agent for controlling cell signal transduction which agent is made of the nucleic acid as claimed in any of claims 1 to 8.

10. The controlling agent as claimed in claim 9, wherein the nucleic acid is an RNA.

12. A method of controlling cell signal transduction using the nucleic acid as claimed in any of claims 1 to 8.

13. The method as claimed in claim 12, wherein the nucleic acid is an RNA.

14. A pharmaceutical composition containing the nucleic acid as claimed in any of claims 1 to 8.

15. The pharmaceutical composition as claimed in claim 14, which composition is used for treating cancers or inflammatory diseases.

16. A method of selecting an RNA having an ability of specific binding to a target protein of Ras, which comprises selecting the RNA having the ability of specific binding to the target protein of Ras from an RNA pool having various base sequences.

17. The method as claimed in claim 16, wherein the RNA of the RNA pool having various base sequences is an RNA comprising 20 to 300 bases.

18. The method as claimed in claim 16 or 17, wherein the

target protein of Ras is Raf-1.

CLAIMS

1. A nucleic acid capable of being specifically bound to a target protein of Ras.

2. The nucleic acid as claimed in claim 1, which nucleic acid is an RNA.

3. The nucleic acid as claimed in claim 1 or 2, which nucleic acid is specifically bound to a Ras binding domain of the target protein of Ras.

4. The nucleic acid as claimed in any of claims 1 to 3, wherein the target protein of Ras is Raf-1.

5. The nucleic acid as claimed in claim 4, which nucleic acid is an RNA that is specifically bound to a Ras binding domain (RBD) of Raf-1.

6. The nucleic acid as claimed in any of claims 2 to 4, wherein the RNA is an RNA containing at least any one of base sequences of sequence Nos. 1 to 28 of Sequence Listing or a base sequence in which at least one base thereof is deleted and substituted with another base and/or at least one base is added.

7. (amended) The nucleic acid as claimed in claim 6, wherein the RNA is an RNA containing at least any one of base sequences of sequence Nos. 1 to 8 or sequence Nos. 25 to 28 of Sequence Listing or a base sequence in which at least one base thereof is deleted and substituted with another base and/or at least one base is added.

8. A nucleic acid having a complementary base sequence

to the nucleic acid as claimed in claim 6 or 7.

9. An agent for controlling cell signal transduction which agent is made of the nucleic acid as claimed in any of claims 1 to 8.

10. The controlling agent as claimed in claim 9, wherein the nucleic acid is an RNA.

11. (added) A method of controlling cell signal transduction using the nucleic acid as claimed in any of claims 1 to 8.

12. (amended) The method as claimed in claim 11, wherein the nucleic acid is an RNA.

13. (amended) A pharmaceutical composition containing the nucleic acid as claimed in any of claims 1 to 8.

14. (amended) The pharmaceutical composition as claimed in claim 13, which composition is used for treating cancers or inflammatory diseases.

15. (amended) A method of selecting an RNA having an ability of specific binding to a target protein of Ras, which comprises selecting the RNA having the ability of specific binding to the target protein of Ras from an RNA pool having various base sequences.

16. (amended) The method as claimed in claim 15, wherein the RNA of the RNA pool having various base sequences is an RNA comprising 20 to 300 bases.

17. (amended) The method as claimed in claim 15 or 16,

wherein the target protein of Ras is Raf-1.

18. (deleted)

ABSTRACT

A novel nucleic acid (aptamer) which binds specifically to the target protein of Ras, more particularly, a novel RNA aptamer which binds specifically to Raf-1; a method for screening an RNA capable of binding specifically to the target protein of Ras which comprises selecting an RNA capable of binding to the target protein of Ras from a pool of RNAs having various base sequences; a method for regulating the signal transduction causing the proliferation or differentiation of cells by using the above-described nucleic acid; and medicinal compositions with the use of the same.

PCR primer 1:
5' - GCCGGAATTCTAATACGACTCACTATAGGAGATCAGAAATAAACGCTCAA-3',
EcoRI T7 promoter

PCR primer 2:
5' -CGCCCTGCAGGGGCTCATGTCGAA-3',
*Pst*I

1/4

RNA clone ^a	Sequence ^b	RNA Bound (%) ^c
Group 1		
21.01 (6)	CUGAUCAAUGGCGUACAAGGAUUCGUUCUCUAUAAACCAAAACCCUUACCCCUUGGACUGA	42
21.02	CUGAUCAAUGGCGUACAAGGAUUCGUUCUCUAUAAACCAAAACCCUUACCCCUUGGACUGA	
21.03 (4)	CUGAUCAAUGGCGUACAAGGAUUCGUUCUCUAUAAACCAAAACCCUUACCCCUUGGACUGC	
21.04	CUGAUCAAUGGCGUACAAGGAUUCGUUCUCUAUAAACCAAAACCCUUACCCCUUGGACUGC	46
21.05	CUGAUCAAUGGCGUACAAGGAUUCGUUCUCUAUAAACCAAAACCCUUACCCCUUGGACUGC	
21.06	CUGAUCAAUGGCGUACAAGGAUUCGUUCUCUAUAAACCAAAACCCUUACCCCUUGGACUGU	46
21.07	UUAGACUCAAUGGCGUACAAGGAUUCGUUCUCUAUAAACCAAAACCCUUACCCCUUGGACUG	12
21.08	UUAGAAGUUCGUACAAGGAUUCG--AUCAUAAACCCGAAGUUUUUAAACACUCUUUACCUGUA	
Group 2		
21.09	UCGAGUCCACGAACAUAUAUUAUUGAACAACUUCAGCACCGCAACAUGCUUAGUACUAUCC	3
21.10	UAUUACCAUAGCCUUGAGGUAAACAUAUUUAGCACACCUGAAUACACGAACUAUGAACUCA	0.2
21.11 (2)	CUUGAGCCAAUUAAAGAUUUACAACAAGAACAUAGAACGUGACAGCGGAUAUAUAACGA	
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Figure 2

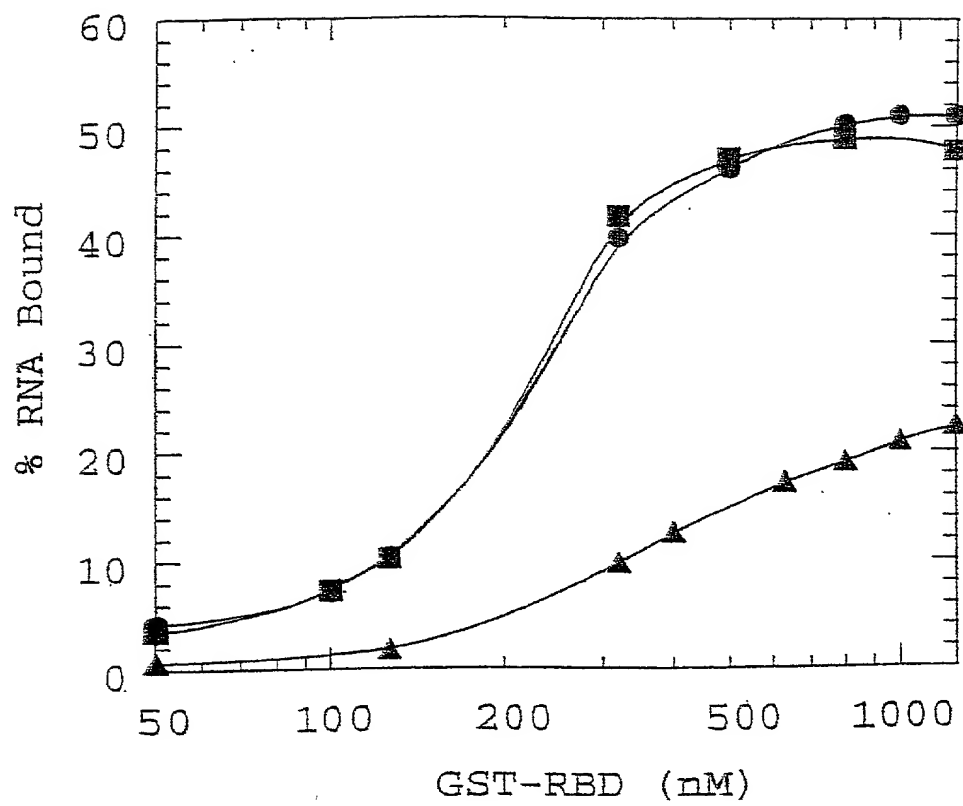


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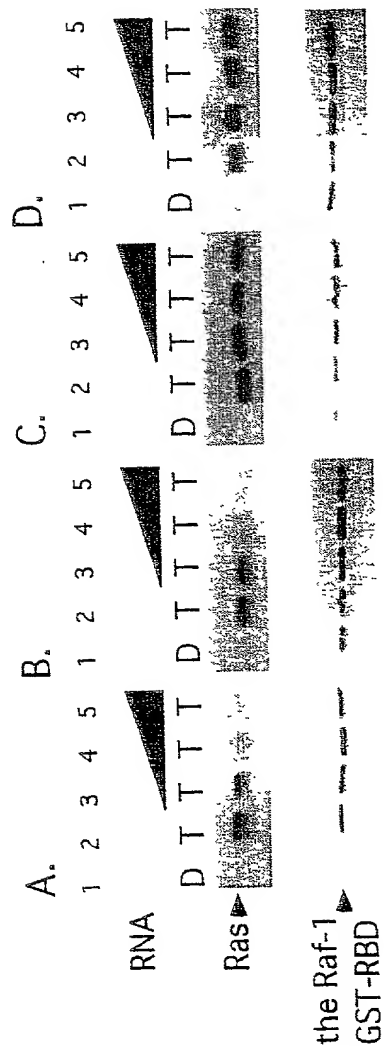


Figure 4

Declaration and Power of Attorney for Patent Application
English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NUCLEIC ACID CAPABLE OF BINDING SPECIFICALLY TO Ras TARGET PROTEIN

the specification of which

(check one)

☐ is attached hereto.
☒ was filed on April 10, 2000 as United States Application No. or PCT
Application No. 09/529,397
and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)	Priority Not Claimed
<u>10-242596/1998</u> (Number)	<u>Japan</u> (Country)
<u>14 August 1998</u> (Day/Month/Year Filed)	<input type="checkbox"/>
<u>10-333284/1998</u> (Number)	<u>Japan</u> (Country)
<u>24 November 1998</u> (Day/Month/Year Filed)	<input type="checkbox"/>
_____ (Number)	_____ (Country)
_____ (Day/Month/Year Filed)	<input type="checkbox"/>

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

I hereby claim the benefit under 35 U.S.C. Section 120 of the United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark office all information known to me to be material to patentability as defined in Title 37, C.F.C., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

PCT/JP99/04399
(Application Serial No.)

August 13, 1999
(Filing Date)

Pending
(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

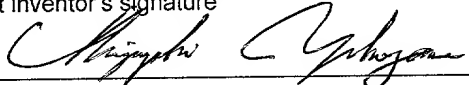
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George W. Neuner	Reg. No. <u>26,964</u>	Robert L. Buchanan	Reg. No. <u>40,927</u>
Linda M. Buckley	Reg. No. <u>31,003</u>	David E. Tucker	Reg. No. <u>27,840</u>
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William J. Daley, Jr.	Reg. No. <u>35,487</u>	Kerri Pollard Schray	Reg. No. <u>47,066</u>

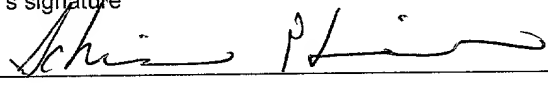
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cgccctgcag gggcctcatg tcgaa

25

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<212> DNA
<213> Artificial Sequence
<220>
<221> primer bind
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45

<210> 60
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<221> primer bind

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catatgcctt agcgacagca agcttctgc

29